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SEX PHEROMONE OF THE CABBAGE LOOPER: REACTIONS WITH ANTENNAL PROTEINS *IN VITRO**

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Abstract—In the presence of (*Z*)-7-dodecen-1-ol acetate, the sex attractant of the cabbage looper, *Trichoplusia ni*, soluble protein from male antennae showed a time-dependent difference spectral absorbance at 280 nm. The change was associated with the enzymatic conversion of the pheromone to (*Z*)-7-dodecen-1-ol, a potent inhibitor of behavioural responses to the pheromone. In contrast, the response obtained with the inhibitor was indicative of non-enzymatic binding to specific protein(s) in the fraction. GLC analyses of the relative rates of enzymatic hydrolysis of the pheromone by the antennae, haemolymph, and legs revealed 33·9, 10·1, and 6·5 per cent conversion per hour, respectively. These results may provide an insight into the fate of a pheromone in the olfactory process of this insect; however, the significance of the reaction with the inhibitor is not yet known.

INTRODUCTION

MANY theories have been proposed to explain the mechanism of qualitative discrimination of odours by olfactory receptors (RODERICK, 1966), but all assume that the odorant molecule must come into the close proximity of, if not contiguity with, the receptor transducer (KLOPPING, 1971). Moreover, studies of the interactions of chemical stimulants with the antennae of insects indicate that a proteinaceous material is involved somewhere in the transducer mechanism. For example, binding of naphthoquinone feeding inhibitors to sulphydryl groups of select antennal proteins was demonstrated in the American cockroach, *Periplaneta americana* (NORRIS *et al.*, 1970; FERKOVICH and NORRIS, 1972). RIDDIFORD (1970) observed presumptive ^{3}H-pheromone binding in soluble proteins eluted from antennae of so-called silkmoths, *Antheraea pernyi* Guér. Also, the data of KASANG and KAISSLING (1972) indicated that an enzymatic mechanism may be involved in the olfactory process of *Bombyx mori* because adsorbed bombykol, (*E*)-10,(*Z*)-12-hexadecadien-1-ol, was progressively degraded into acid and ester.

In a preliminary study, FERKOVICH *et al.* (1972) demonstrated that soluble protein(s) from antennae, legs, and haemolymph of the cabbage looper, *Trichoplusia ni* (Hübn.), reacted with various chemicals that are known to modify the

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behaviour of the insect. Thus, a protein(s) was involved at some point in the olfactory process. However, the significance of the reactions of antennal protein(s) and behaviourally active chemicals was not readily understood. We therefore examined the reactions in greater detail and attempted to further localize the source of the reactive protein(s) in the antennae.

The cabbage looper was again chosen for the study for several reasons: (1) the behavioural responses of this insect to the pheromone and to various related chemicals have been well studied (TOBA *et al.*, 1970); electrophysiological studies have been made of all the chemicals (MAYER, 1973a, b); and (3) all the chemicals and pheromones identified as related to the cabbage looper were available in high purity.

MATERIALS AND METHODS

Preparation of soluble protein

Unless otherwise stated, all excisions and homogenizations were carried out in 0·5 M sucrose buffered with 0·05 M Tris–HCl, pH 7·5. All studies of reactions were performed in 0·05 M Tris–HCl, pH 7·5.

Antennae and legs (*ca.* 25 mg wet wt.) of live cabbage looper males were excised without anaesthesia and placed separately in 4 ml of sucrose-buffer, and homogenized with a motor-driven, Teflon-glass homogenizer in an ice-bath. The homogenate was filtered through glass wool to remove cuticular debris and centrifuged at 20,000 ***g*** for 45 min at 4°C; then the supernatant was decanted and saved. A 2 ml aliquot of the antennal supernatant was centrifuged at 105,000 ***g*** for 2 hr to remove the subcellular particles, and the supernatant was saved. Haemolymph was collected in glass capillaries at the cervical membrane, diluted with an equal volume of buffered-sucrose, and centrifuged at 20,000 ***g*** for 45 min; the resultant supernatant was saved. Protein contents of the supernatants were determined by the method of LOWRY *et al.* (1951).

Interaction of pheromone with protein preparations monitored by u.v. difference spectroscopy

A method of u.v. difference spectroscopy patterned after that of NORRIS *et al.* (1970, 1971) was used to compare the binding affinity of the antennal, leg, and haemolymph supernatants for the pheromone, (*Z*)-7-dodecen-1-ol acetate (BERGER, 1966). Then the relative binding affinities of these proteins from each source for the pheromone were compared with those for the alcohol, (*Z*)-7-dodecen-1-ol, an inhibitor of behavioural responses to the pheromone (TUMLINSON *et al.*, 1972), and for dodecan-1-ol acetate, a saturated behaviourally inactive analogue of the pheromone (TOBA *et al.*, 1970).

Measurements of the change in absorbance induced by the chemical stimulants were made at 280 nm. Interactions with the protein preparations were recorded with a Gilford Model 2000 single beam recording photometer coupled to a Beckman DU monochromator. Baselines (zero absorbance as a function of 280 nm) were attained by electronically neutralizing the initial absorbance of the chemical and protein preparations with the absorbance control system. Full-scale sensitivity of

the recording system was set at 0 to 0·5A. The temperature of the cell compartment was maintained at 20°C.

Stock solutions of the treatment chemicals dispersed in double-distilled water by sonication with a Biosonik III at minimum intensity (*ca.* 1 W/cm² of the probe) for 1 min were used immediately after preparation. Usually, either 5 or 25 μl of the sonicated stock solutions were added to 100 μg of protein preparation in 1·0 ml buffer to produce final concentrations of 5×10^{-4} and $1{\cdot}25 \times 10^{-3}$ M, respectively.

The synthetic pheromone was 95+ per cent pure and contained no measurable inhibitor by GLC analysis; the alcohol (inhibitor) was 99+ per cent pure; and the saturated pheromone analogue was 98+ per cent pure by GLC analysis.

In some cases, spectral measurements were made with a Perkin–Elmer Model 356 u.v.-visible spectrophotometer in the split beam mode to determine the influence of the pheromone and the inhibitor on the spectra of the protein alone. Baselines were obtained with either the pheromone or the inhibitor ($1{\cdot}5 \times 10^{-3}$ M) in both the reference and sample cell in 0·05 M Tris–HCl, pH 7·5. The protein solution (42 μg/ml) was then added to the sample cell, and the temporal change in the spectrum over 200–350 nm was measured with the 0–0·3 A scale.

The specificity of the interaction between the pheromone and the antennal protein(s) was determined by comparing the changes in u.v. absorbance of the antennal protein (100 μg/ml) and bovine serum albumin (100 μg/ml) upon admixture with the pheromone ($1{\cdot}25 \times 10^{-3}$ M). The proteinaceous nature of the antennal components was confirmed by heating the aliquots of the antennal supernatant (100 μg/ml) at 64°C or incubating them with 100 μg trypsin (pH 7·5) at 20°C for 20 min before they were mixed with the pheromone.

Preparation of sonicated antennal protein

The antennal protein was prepared by excising 100 pairs of male moth antennae into a 5 ml beaker (in ice) containing 2·0 ml of Tris–sucrose and sonicating in an ice-bath in a Ladd, Model T-586 (82 kHz) ultrasonic cleaner at full power for 10 min. The supernatant was decanted and saved (fraction A). Several of the antennae were examined by scanning electron microscopy; the others were homogenized in buffered-sucrose (fraction B). Both fractions A and B were then centrifuged at 105,000 *g* for 1 hr, and the resultant supernatants were saved.

Gas chromatographic analysis of reaction of pheromone with antennal protein

Sonicated pheromone in distilled water (final concentration $2{\cdot}02 \times 10^{-2}$ M) was incubated with 2·0 ml of soluble antennal protein (100 mg wet wt./ml). Aliquots of 0·2 ml were collected during the reaction and were each extracted with 0·5 ml of anhydrous ether. One 0·2 ml sample was held as a control in a boiling water-bath for 15 min before a 60 min incubation and then extracted. These ether extracts were then chromatographically analysed by using a Varian Aerograph Model 2100 gas chromatograph with a flame ionization detector that was equipped with glass columns (1·8 m by 2 mm i.d.) packed with either 5% Carbowax 20M on

Chromosorb W or 3% SE-30 on 100/120 Varaport. The temperature of the columns was maintained at 170°C; the nitrogen carrier flow rate was 30 ml/min.

A GLC analysis of the interaction of haemolymph and leg protein with pheromone was also performed as described. Thus, 0·2 ml aliquots were collected after a 60 min incubation, and the relative percentage of pheromone converted to alcohol by each protein source was calculated.

RESULTS

Comparison of interactions of antennal proteins with chemical stimulants by u.v. difference spectroscopy

The change observed in negative difference absorbance at 280 nm obtained with the single beam spectrophotometer was used to study the rates and types of reactions of the Tris-soluble antennal proteins. The negative change in absorbance increased with increasing concentrations of antennal proteins and pheromone (Fig. 1). When the rate of change diminished after 1 hr, it could be reactivated by adding 25 μl of pheromone (arrow, Fig. 1). This reaction was indicative of enzymatic rather than non-enzymatic binding because a gradual change in absorbance is

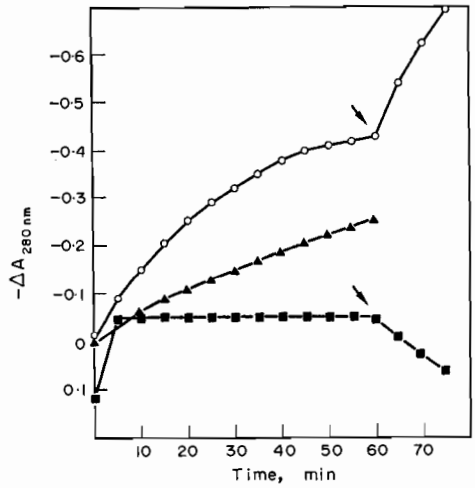

Fig. 1. Change in difference spectral absorbance at 280 nm: after the addition of $1{\cdot}25 \times 10^{-3}$ M pheromone to 25 (—▲—▲—) and to 100 μg (—○—○—) protein/ml of antennal supernatant vs. change when $1{\cdot}25 \times 10^{-3}$ M inhibitor was added to 100 μg (—■—■—) protein/ml of antennal supernatant. Note the responses when a second aliquot of each chemical was added after 60 min (arrow).

characteristic of chemical (enzymatic) reaction (Ash, 1968). The inhibitor ($1{\cdot}25 \times 10^{-2}$ M) reacted in an opposite way. A rapid initial positive increase in absorbance (not shown in Fig. 1) was followed by the formation of a negative $-\Delta A$ activity plateau that was stable for over 1 hr (Fig. 1), an indication of non-enzymatic binding. Addition of inhibitor caused another but slower positive increase in absorbance. This

type of result was suggestive of proteins undergoing reversible conformational transitions from one folded state to another upon binding to the inhibitor (LASKOWSKI, 1970). Incubation of the antennal preparation (100 μg protein/ml) with the inhibitor ($1{\cdot}25 \times 10^{-3}$ M) for 15 min before the addition of the pheromone ($1{\cdot}25 \times 10^{-3}$ M) did not prevent the change in absorbance caused by the addition of the pheromone.

Comparison of reactions of haemolymph and leg proteins by u.v. difference spectroscopy

The experiments just cited were duplicated with haemolymph and leg proteins to determine whether the types and rates of reactions were specific for antennal proteins. The data in Fig. 2 indicate that the absorbance changes observed with

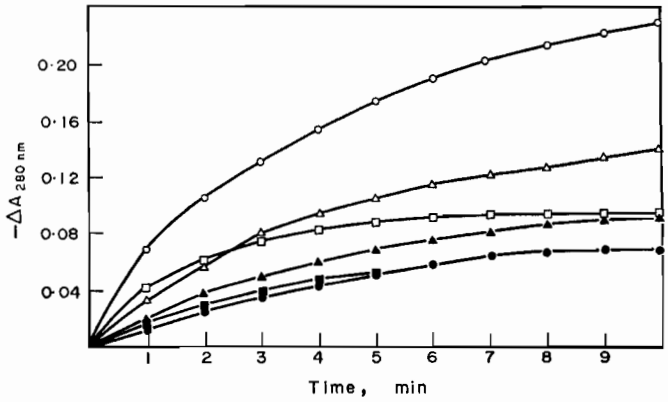

FIG. 2. Change in difference spectral absorbance at 280 nm: after the addition of the pheromone (5×10^{-4} M) to fractions (100 μg protein/ml) of the antennae (—○—○—), haemolymph (—□—□—), and leg (—△—△—) or of dodecyl acetate (5×10^{-4} M) to fractions of antennae (—●—●—), haemolymph (—■—■—), and leg (—▲—▲—).

antennal preparations were also obtained with the haemolymph and legs. However, the rate of the pheromone-protein interaction was greatest with the antennal preparations and lowest with the saturated analog of the pheromone. Thus, the reactive protein(s) were reasonably specific for the pheromone, and the reaction was predominant in the antenna.

Definite proof of specificity awaits purification of the reactive protein(s) and concomitant tests with closely related isomers and analogues of the pheromone.

Confirmation of proteins as the reactant materials

Removal of the microsomal fraction did not decrease the activity of the soluble protein fraction (Fig. 3) though it showed slight activity. Incubation with trypsin and heating of the antennal supernatant at 64°C destroyed the reactivity of the supernatant (Fig. 4). No measurable change in absorbance resulted when bovine

serum albumin was mixed with the pheromone. Therefore, the monitored change in absorbance was a specific interaction of the pheromone with a Tris-soluble non-particulate protein(s) from the antennae and was not a general reaction with non-specific proteins.

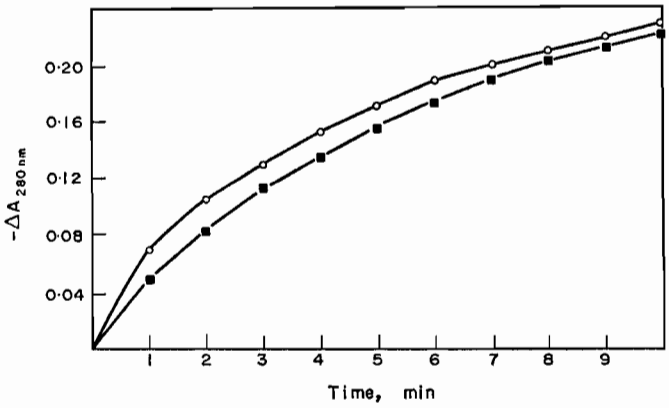

FIG. 3. Change in difference spectral absorbance at 280 nm: after the addition of the pheromone (5×10^{-4} M) to antennal fraction (100 μg protein/ml) that did (—○—○—) or did not (—■—■—) contain the microsomal fraction.

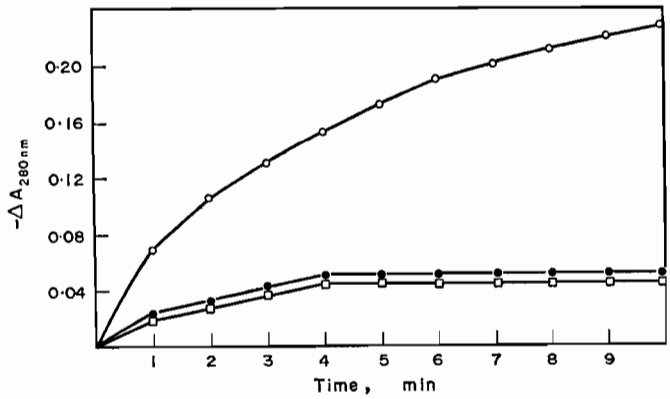

FIG. 4. Change in difference spectral absorbance at 280 nm: after the addition of pheromone (5×10^{-4} M) to antennal fraction (100 μg protein/ml) that was untreated (—○—○—); heated at 64°C for 30 min (—●—●—); or incubated with 100 μg trypsin for 30 min (—□—□—).

Pheromone degradation monitored by GLC

Evidence that the pheromone was enzymatically hydrolysed to the behaviourally inhibitory alcohol was reported previously (FERKOVICH *et al.*, 1972). Quantitative

GLC analyses of the reaction (Table 1) confirmed the enzymatic nature of the interaction only suspected from u.v. difference data. The heat-treated sample incubated with the pheromone gave only one peak with a retention time identical to that of the pheromone (retention times 4·71 min on the Carbowax 20M column and 2·24 min on the SE-30 column). All other incubated samples had two peaks, one that represented the pheromone and a second one that had a retention time identical to that of authentic (*Z*)-7-dodecen-1-ol (5·88 and 1·29 min, on Carbowax 20M and SE-30 columns, respectively).

TABLE 1—RATIO OF PEAK HEIGHT OF PHEROMONE TO PEAK HEIGHT OF ALCOHOL DURING INCUBATION OF PHEROMONE (FINAL CONCENTRATION $2{\cdot}02 \times 10^{-2}$ M) WITH 2·0 ml ANTENNAL HOMOGENATE (100 mg wet wt./ml)

Incubation time (min)	Ratio of peak height of pheromone to peak height of alcohol
1	93·0
5	47·5
9	15·5
15	8·7
30	4·0
45	2·2
60	1·7

Subsequent tests of the relative rates of enzymatic hydrolysis by various protein fractions revealed 33·9, 10·1, and 6·5 per cent conversions/60 min by antennae, haemolymph, and legs, respectively.

u.v. absorption studies of interactions of pheromone and inhibitor with protein obtained from sonicated whole antennae

According to SCHNEIDER (1970), the dendritic nerve endings in the chemosensory sensilla of the antennae are bathed by a fluid termed the 'sensillum liquor'. Also, RIDDIFORD (1970) reported the binding of a ^{3}H-pheromone to protein(s) subject to constant resynthesis and suggested that it was localized in the pore tubule system of the olfactory chemosensilla; however, it was not clear that the substances were receptor proteins. These studies therefore suggested that we might tentatively establish the source of the reactive proteins by sonicating male cabbage looper antennae to fracture the tips of the sensilla so the protein in the sensilla would be released into solution. Thus the interactions of the inhibitor and the pheromone with the protein obtained by this method (fraction A) were compared with the interactions with the soluble protein supernatant (fraction B) obtained by homogenization of the remainder of the antenna (minus the protein released by the sonication treatment).

In the u.v. difference spectroscopy technique described for the single beam spectrophotometer, the change in absorbance recorded during the pheromone-protein interaction was attributed to perturbations in the chromophores of the

proteins (LASKOWSKI, 1970). In addition, turbidity due to the dispersed droplets of the pheromone or inhibitor visibly decreased during interaction of the pheromone and inhibitor with the protein(s). Consequently, changes in the light scattering properties of the chemicals upon binding the proteins may also have contributed to the changes in absorption (TELLER, 1972). In this case, spectral measurements were made with a dual beam instrument (engineered to measure the spectra of turbid and light scattering solutions) to observe the influence of the pheromone and inhibitor on the spectra of the protein(s) alone. Thus, binding of the chemical stimulants to the proteins was followed by the change in the spectrum of protein(s). Such changes in u.v. absorption spectra of proteins upon binding of molecules have been reported by several investigators (DONOVAN, 1969; FISHER *et al.*, 1969; HASSING and GOLDSTEIN, 1970; LASKOWSKI, 1970).

Examination of the antennae by scanning electron microscopy revealed that the sonication in Tris–sucrose had broken many of the sensilla tips. Fractions A and B contained 800 and 170 μg/ml of protein, respectively. Also, the absorption ratios of 280 nm/260 nm fractions A and B were 1·22 and 0·69, respectively, an indication of predominance of protein in fraction A and of nucleic acids and microsomes in fraction B. Therefore, sonication of the antennae probably released the majority of the soluble protein (presumably the sensillum liquor) because fraction A contained over four times as much protein as fraction B. The results also indicated that a 1 hr centrifugation at 105,000 g in 0·5 m sucrose in Tris buffer was not entirely adequate to sediment the microsomal particles of fraction B.

The u.v. absorption spectra of fraction A in the presence or absence of the pheromone is shown in Fig. 5. The absorbance of the spectrum (maximum at 285 nm) varied with time during interaction with the pheromone. The initial influence produced a bathochromic shift and a reduction in absorbance at all wavelengths between 230 and 350 nm. However, the absorbance of the entire spectrum as a function of the protein interaction with the pheromone varied non-linearly with time and reached the original absorbance of the fraction when the pheromone was absent. After 25 min, only a barely measurable change in absorbance was noted.

The initial reaction of the inhibitor with fraction A produced a decrease in absorbance from 230 to 350 nm without an apparent shift of the spectrum* (Fig. 6). However, like the reaction with the pheromone, the reaction with the inhibitor resulted in an initial depression of the absorbance spectrum and thereafter, a consistent increase in absorbance to 20 min at which time it has surpassed the original absorbance of the protein and then stabilized.

The u.v. absorption of fraction B (maximum at 257 nm) in the presence and absence of the pheromone are shown in Fig. 7. The initial reaction of the pheromone with this fraction reduced the absorbance of the entire spectrum relative to the protein alone, and this type of reaction continued through 10 min. Thereafter,

* TINOCO (1960), in a discussion on ligand binding to proteins, used the term hypochromicity to describe the phenomenon of a proportional change in absorbance at all wavelengths without a shift in wavelength.

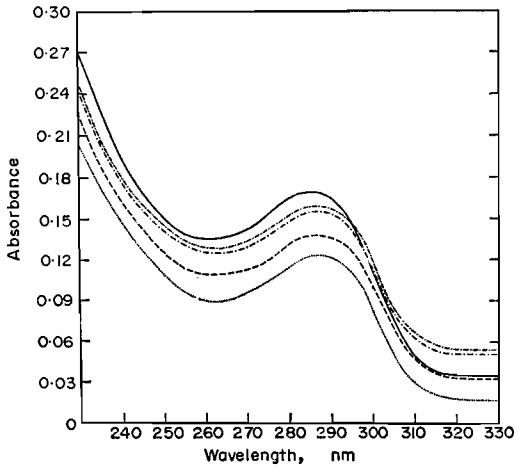

FIG. 5. U.v. absorption spectrum of: fraction A (42 μg protein/ml) in 0·05 M Tris–HCl, pH 7·5, in sample cell vs. Tris–HCl (alone) in the reference cell, ——; fraction A with pheromone Tris–HCl in sample cell vs. Tris–HCl + $1{\cdot}5 \times 10^{-3}$ M pheromone in reference cell after 0 min,- - - - -; 10 min, – / — / –; 20 min, – – –; 25 min — - —.

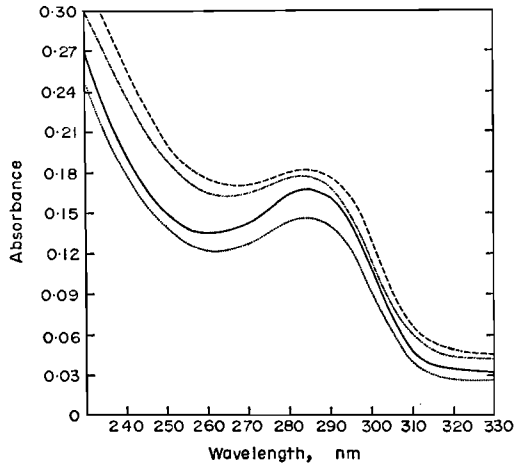

FIG. 6. U.v. absorption spectrum of: fraction A (42 μg protein/ml) in 0·05 M Tris–HCl, pH 7·5, in sample cell vs. Tris–HCl alone in the reference cell, ——; fraction A (42 μg protein/ml) + $1{\cdot}5 \times 10^{-3}$ M inhibitor in Tris–HCl in sample cell vs. Tris–HCl + $1{\cdot}5 \times 10^{-3}$ M inhibitor in reference cell after 0 min,- - - - -; 10 min, – / — / –; 20 min, – – –.

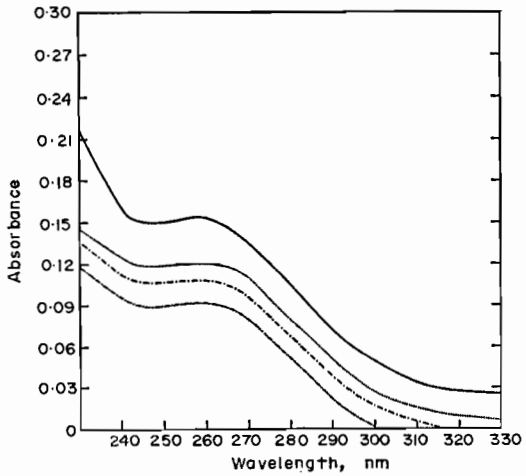

FIG. 7. U.v. absorption spectrum of: fraction B (42 μg protein/ml) in 0·05 M Tris–HCl, pH 7·5 in sample cell vs. Tris–HCl alone in the reference cell ——; fraction B (42 μg protein/ml) + $1{\cdot}5 \times 10^{-3}$ M pheromone in Tris–HCl in sample cell vs. Tris–HCl + $1{\cdot}5 \times 10^{-3}$ M pheromone in reference cell after 0 min, - - - - - -; 5 min, — - —; 10 min, – / — / –.

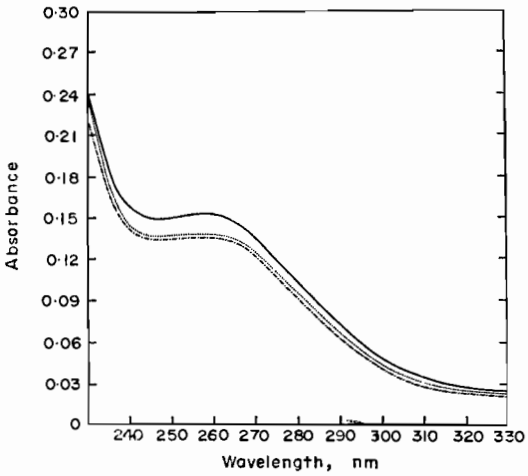

FIG. 8. U.v. absorption spectrum of: fraction B (42 μg protein/ml) in 0·05 M Tris–HCl, pH 7·5 in sample cell vs. Tris–HCl alone in the reference cell ——; fraction B (42 μg protein/ml) + $1{\cdot}5 \times 10^{-3}$ M inhibitor in Tris–HCl in sample cell vs. Tris–HCl + $1{\cdot}5 \times 10^{-3}$ M inhibitor in reference cell after 0 min, - - - - - -; 5 min, – / — / –; 10 min, – / — / –.

the decrease was minimum on the 0 to 0·3 A scale expansion of the spectrophotometer. Microsomal hydrolysis may thus explain the reaction of the pheromone with fraction B. The inhibitor produced only a slight initial reduction in absorbance of the entire spectrum, and no change after 5 min; and it stabilized after 10 min (Fig. 8).

DISCUSSION

The fate of a sex pheromone in an olfactory sensillum subsequent to the stimulation of the neuron has been the subject of considerable speculation (SCHNEIDER, 1970; KAISSLING, 1971). Our results point strongly to an enzymatic mechanism in the cabbage looper that degrades the pheromone. However, the attractant is converted to a potent behavioural inhibitor—the alcohol; therefore, an intriguing question is raised concerning the possible function of such a system in olfaction in this insect and in other species that have long-chain hydrocarbon acetates as pheromones. KASANG and KAISSLING (1972) reported that radiolabelled bombykol was degraded to acidic and ester fractions by the antennae and other body parts of male and female *Bombyx mori*. In earlier studies SCHNEIDER (1970) suggested that such processes in the silkworm may be related to chemoreception because the antennae may degrade the odorant following stimulation. He further suggested that metabolic degradation of the pheromone would explain why female moths are only attractive as long as their lure glands are everted because pheromone absorbed on the body surface is rapidly degraded. That female cabbage loopers, too, have been reported to actively degrade the pheromone to the alcohol (FERKOVICH *et al.*, 1972) suggests a more profound rôle of the metabolites of the pheromone in the olfaction and behaviour of both sexes of this insect.

Comparison of the reactions of antennal protein with the pheromone and inhibitor by u.v. difference spectroscopy using the single beam spectrophotometer and GLC analyses made it possible to distinguish between enzymatic binding of the pheromone and non-enzymatic binding (complexation) by the inhibitor to antennal proteins. The pheromone and inhibitor probably bind to different sites on the same receptor protein or to different receptor protein(s) because preincubation of the protein(s) with inhibitor before the addition of the pheromone did not prevent the characteristic change in absorbance induced by addition of the pheromone. Further elucidation of the kinetics of the reactions and the reactive sites (e.g. sulphydryl groups were reactive sites in select antennal proteins for naphthoquinone feeding inhibitors (NORRIS *et al.*, 1970; FERKOVICH and NORRIS, 1972) will require purification of the active protein(s).

The interactions of the pheromone and inhibitor with proteins from fractions A and B obtained with the dual beam spectrophotometer revealed some differences that were instructive. First, enzymatic degradation of the pheromone probably occurred in both fractions, but the protein spectra indicated at least two different enzymes. Second, the alcohol produced a relatively greater change in absorbance in fraction A than in fraction B (Figs. 6, 8) though a minor change occurred in the spectrum of fraction A. Indeed, the probable reason for the variable effect of

pheromone on the spectrum of fraction A was the presence of other protein(s) binding with the alcohol as it was converted from the pheromone. Also, the slight reaction of the saturated analogue of the pheromone with the antennal proteins indicated a greater specificity of these proteins than is generally ascribed to esterases (DIXON and WEBB, 1964). Therefore, the result implies a more than passing relationship to olfactory discrimination in this insect. The further importance of these observations is that the alcohol-binding protein(s) apparently is localized in the sensilla (fraction A) and that the reactions of this fraction indeed are those that occur in the sensilla.

Our results would seem to substantiate the stereochemical theory of AMOORE (1964, 1969) which correlates the odour qualities of molecules with their size and shape and chemically reactive sites required to fit specific olfactory receptor sites. Thus, we tentatively conclude that the interactions of the pheromone and alcohol with antennal proteins that we report are somehow necessary for olfactory perception by the cabbage looper.

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